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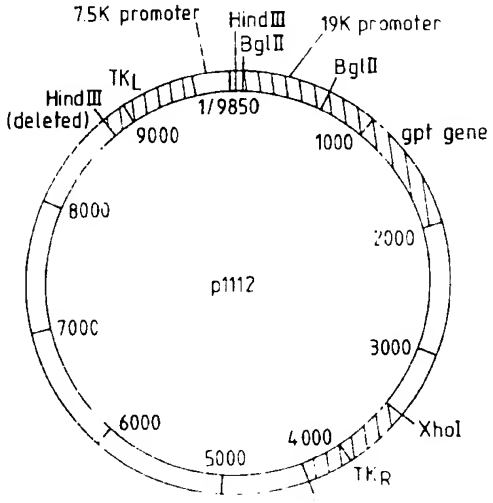
(54) Title: ANTIBODY PRODUCTION IN VACCINIA VIRUS INFECTED CELLS

(57) Abstract

The invention relates to recombinant vaccinia virus capable of expressing on infection of a suitable cell the light chain and/or the heavy chain of an antibody and the use of such a virus in the production of recombinant antibodies. In order to produce the recombinant antibody, cells are cultured which are infected with a recombinant vaccinia virus capable of expression of the light chain and the heavy chain of the antibody or co-infected with recombinant vaccinia viruses capable of expression of the light chain and heavy chain respectively of the antibody



(CDR-grafted) antibody for example a humanised antibody against the CDw52 antigen.

LIGHT CHAIN
AAGCTT TACAGT TACTGAG ACACAGGACCTCACC ATG GGA TGT TAG AAGCTT
met gly cys

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ANTIBODY PRODUCTION IN VACCINIA VIRUS INFECTED CELLS

The present invention relates to recombinant vaccinia virus vectors and to the production of recombinant antibodies using such vectors.

Antibodies or immunoglobulins are proteinaceous bifunctional molecules. One part, which is highly variable between different antibodies, is responsible for binding to an antigen, for example the many different infectious agents that the body may encounter, whilst the second, constant, part is responsible for binding to the Fc receptors of cells and also activates complement. In this way, antibodies represent a vital component of the immune response of mammals in destroying foreign microorganisms and viruses.

The immunisation of an animal with an antigen results in the production of different antibodies with different specificities and affinities. An antiserum obtained from the immunised animal will, therefore, be heterogeneous and contain a pool of antibodies produced by many different lymphocyte clones. Antibodies thus obtained are referred to as polyclonal antibodies and this polyclonal nature has been a major drawback in the use of antibodies in diagnostic assays and in therapeutic applications.

A major step forward occurred in 1975 when Kohler and Milstein (Nature, 1975, 256, 495-497) reported the successful fusion of spleen cells from mice immunized with an antigen with cells of a murine myeloma line. The resulting hybrid cells, termed hybridomas, have the properties of antibody production derived from spleen cells and of continuous growth derived from the myeloma cells. Each hybridoma synthesizes and secretes a single antibody to a particular determinant of the original antigen. To ensure that all cells in a culture are identical, i.e. that they contain the genetic information [illegible] one.

[illegible] produce homogeneous or monoclonal antibodies

The advantages of hybridoma technology are profound. Because many hybrids arising from each spleen are screened for their potential to produce antibodies to the antigen of interest and only a few are selected, it is possible to immunize with impure antigens and yet obtain specific antibodies. The immortality of the cell line assures that an unlimited supply of a homogeneous, well-characterised antibody is available for use in a variety of applications including in particular diagnosis and immunotherapy of pathological disorders. Unfortunately, the usefulness of such monoclonal antibodies in a clinical setting can be severely hampered by the development of human anti-mouse antibodies - an anti-globulin response - which may interfere with therapy or cause allergic or immune complex hypersensitivity. This has led to the development of humanised antibodies.

An antibody molecule is composed of two light chains and two heavy chains that are held together by interchain disulphide bonds. Each light chain is linked to a heavy chain by disulphide bonds and the two heavy chains are linked to each other by disulphide bonds. Each heavy chain has at one end a variable domain followed by a number of constant domains, and each light chain has a variable domain at one end and a constant domain at the other end. The light chain variable domain is aligned with the variable domain of the heavy chain. The light chain constant domain is aligned with the first constant domain of the heavy chain. The remaining constant domains of the heavy chains are aligned with each other. The constant domains in the light and heavy chains are not involved directly in binding the antibody to the antigen.

The variable domains of each pair of light and heavy chains form the antigen binding site. They have the same general structure with each

conformation and the CDRs form loops connecting, and in some cases

comprising part of, the beta-sheet structure. The CDRs are held in close proximity by the framework regions and, with the CDRs from the other domain, contribute to the formation of the antigen binding site.

In the use of murine monoclonal antibodies, the induction of an human anti-mouse antibody response is due to the murine origin of the constant domains and four framework regions. This problem has therefore been addressed by the development of modified antibodies of two basic types. The first type, referred to as chimeric antibodies, is where the murine constant domains only are replaced by equivalent domains of human origin (Morrison et al, P.N.A.S., 1984, 81, 6851-6855; Boulianne et al, Nature, 1985, 314, 268-270; and Neuberger et al, Nature, 1985, 314, 268-270). The second type, referred to as humanised antibodies, is where the murine constant domains and the murine framework regions are all replaced by equivalent domains and regions of human origin. As well as being referred to as humanised, this second type of modified antibody is also referred to as a CDR-grafted antibody (Jones et al, Nature, 1986, 321, 522-525; and Riechmann et al, Nature, 1988, 332, 323-327).

To generate sufficient quantities of antibody for full clinical investigation, it is desirable to utilize an efficient recombinant expression system. Since myeloma cells represent a natural host specialized for antibody production and secretion, cell lines derived from these have been used for the expression of recombinant antibodies. Often, complex vector design, based around immunoglobulin gene regulatory elements, is required, and final expression levels have been reported which are highly variable (Winter et al, Nature, 1988, 332, 323-327; Weidle et al, Gene, 1987, 60, 205-216; Nakatani et al, Bio/Technology, 1989, 7, 805-810; and Gillies et al, Bio/Technology, 1989, 7, 799-804).

Acad Sci USA 1988, [illegible] however yields are generally low

and it is difficult to establish stable cell lines. E. coli has been used to express Fv fragments (Skerra & Plukthun, Science, (1988) 240, 1038-1041) or single chain antigen binding molecules (Bird et al, Science, (1988) 242, 423-426) but entire immunoglobulins have so far not been produced in the system.

The use of vaccinia virus as a vector for expression of foreign genes has been employed for almost a decade. Over 75 different virus gene products and a variety of other proteins including growth factors, cell surface antigens, oncogenes, bacterial structural proteins and enzymes, and protozoan proteins have been expressed in vaccinia for a variety of purposes (Mackett, Seminars in Virology, 1990, 1, 39-42). One advantage of this system is the authenticity of gene products, particularly those requiring processing and post-translational modification such as glycosylation. This may be particularly important for genes of mammalian origin.

It has now been found that vaccinia virus vectors can be used for expression of the light and heavy chains of a recombinant antibody in a suitable host cell and that a proportion of the chains combine within the cell to form a recombinant antibody which is secreted into the medium and can thus be recovered in functional form.

According to one aspect, the present invention provides a recombinant vaccinia virus capable of expressing on infection of a suitable cell the light chain and/or the heavy chain of an antibody.

Recombinant vaccinia viruses containing foreign DNA may be prepared by infecting suitable cells with wild type vaccinia virus and then transfecting the infected cells with a transfer vector containing the foreign DNA, sufficient vaccinia virus DNA to allow for recombination

According to another aspect, the present invention provides a process for the production of a recombinant vaccinia virus as defined above which comprises the steps of:

(i) infecting suitable cells with wild type vaccinia virus;

(ii) transfecting the infected cells with a transfer vector containing DNA encoding the light and/or heavy chain of the antibody under control of a suitable promoter, DNA encoding a selectable marker also under control of a suitable promoter and sufficient vaccinia virus DNA to allow for recombination with the wild type vaccinia virus;

(iii) harvesting vaccinia virus from the transfected cells;

(iv) re-infecting further suitable cells with the harvested vaccinia virus;

(v) selecting re-infected cells by means of the selectable marker; and

(vi) harvesting recombinant vaccinia virus from the selected cells.

Suitable selectable markers for use in the transfer vector include the E. coli guanine phosphoribosyltransferase (gpt) gene which allows for positive selection of recombinant viruses by growth of the infected cells in the presence of mycophenolic acid (Boyle & Coupar, Gene, (1988), 65, 123-128; Falkner & Moss, J. Virol., (1988), 62, 1849-1854).

The transfer vector contains DNA encoding the light chain and/or the heavy chain of an antibody together with a suitable promoter and the selectable marker will also be under control of a suitable promoter. On recombination with the wild type virus a recombinant virus is produced which is capable, on infection of a suitable cell, of expressing light chain and/or heavy chain of the antibody. Suitable promoters include [illegible]

B.N. Fields and D.M. Knipe, Raven Press, New York, 1990, chapter 7.

The recombinant vaccinia virus according to the invention may be used to express an antibody by using the recombinant vaccinia virus to infect suitable host cells, culturing the infected cells and recovering the antibody produced. Where a recombinant vaccinia virus is produced containing DNA capable of expressing only one chain (the light chain or the heavy chain) of an antibody, then it will be necessary to co-infect the host cells with another recombinant vaccinia virus containing DNA capable of expressing the other chain.

Alternatively, a single recombinant vaccinia virus can be produced which is capable of expressing both chains of the antibody. This can be achieved by having both coding sequences under control of the same promoter so that a bi-cistronic mRNA is produced or by using a dual promoter system in which each coding sequence is under control of a separate promoter.

In either case, expression of the two coding sequences in the infected cells is followed by combination of the two chains within the host cell and secretion of a functional antibody. Two important factors in the control of secretion of these molecules are the heavy binding protein (Bip) (Pelham, Biochem. Soc. Trans., (1989), 17, 795-802) and the intracellular concentration of the light chain. Bip binds to the region on heavy chain that is involved in the association with light chain and only light chain with a greater affinity for heavy chain than Bip can displace it and thus allow assembly and secretion of complete antibody molecules. Light chain may also be secreted in the absence of heavy chain and thus has to be synthesised at a sufficient rate to achieve a high enough concentration to displace Bip from the heavy chain.

Construction of a single recombinant vaccinia virus capable of

amounts of two recombinant vaccinia viruses. However it may also be

advantageous to produce a biased expression system such that one chain is synthesised to a higher level than the other. In the system described in more detail in the following examples, heavy and light chain were synthesised at roughly equal levels but it was found that only a portion of the total amount of each chain which was synthesised was actually assembled. The excess heavy chain remained in the cell and the excess free light chain was mostly secreted. In such a case there may be an advantage in adjusting the ratio of one chain to the other, for example to lower the ratio of the light chain to the heavy chain. One way of adjusting the ratio of one chain to the other is to vary the multiplicity of infection of the two viruses. Also there may be an advantage in infecting the cells at different times thereby giving any limiting component a head start, although bearing in mind that cells may become refractory to infection by a second virus if left too long. Further ways of adjusting the ratio of one chain to another may be to use promoters of different strengths or with different temporal requirements.

According to a further aspect, the present invention provides a process for the production of a recombinant antibody which comprises culturing cells which are infected with a recombinant vaccinia virus capable of expression of the light chain and the heavy chain of the antibody or co-infected with recombinant vaccinia viruses capable of expression of the light chain and the heavy chain respectively of the antibody and recovering the antibody from the culture medium.

According to a yet further aspect, the invention provides a cell line capable of expressing a recombinant antibody, characterised in that the cell line is infected with a recombinant vaccinia virus capable of expression of both the light chain and the heavy chain of the antibody or co-infected with recombinant vaccinia viruses capable of expression of the light [illegible]

[illegible] and any suitable cells capable of infection by vaccinia virus can be

used according to the invention both for production of the recombinant vaccinia virus and as host cells for the production of the antibody. Suitable cells include HeLa cells, particularly HeLa (TK-) cells, and B cell lines such as NSO and Namalwa. The fact that vaccinia virus will infect such a wide range of mammalian cells means that the choice of host can be made on the basis of other criteria such as ability to perform appropriate post-translational modification, secretion properties, ability to grow in suspension and lack of requirement for serum in the growth medium. It is particularly advantageous if the cells used for production of the antibody are capable of growth on serum free medium since this greatly simplifies purification of the antibody.

Expression levels of the two chains of the antibody can be enhanced by use of T7 polymerase to amplify the gene under the control of the T7 promoter. The T7 polymerase can be produced either by co-infection with a recombinant vaccinia virus expressing the enzyme (Fuerst et al, Proc. Natl. Acad. Sci. USA, (1986), 83, 8122-8126) or by using a host cell line which constitutively expresses T7 polymerase (Elroy-Stein & Moss, Proc. Natl. Acad. Sci. USA, (1990), 87, 6743-6747). Expression levels can also be enhanced by including a DNA segment, corresponding to part of the 5'-untranslated region of encephalomyocarditis virus, downstream of the T7 promoter (Elroy-Stein et al, Proc. Natl. Acad. Sci. USA, (1989), 86, 6126-6130).

The present invention can be used to produce all kinds of antibodies that generally comprise equimolar proportions of light and heavy chains. It may thus extend to the production of Fab fragments and to bispecific antibodies. However, the invention is most preferably applied to the production of human antibodies or of chimaeric antibodies and humanised (CDR-grafted) antibodies. Particular

Campath-1H. "CAMPATH" is a Registered Trade Mark of the Wellcome group of companies.

Further examples include chimaeric and, in particular, humanised antibodies against various tumour cell marker antigens.

The light and heavy chain genes may constitute genomic DNA or, preferably, cDNA and may be cloned using procedures known in the art (Molecular Cloning: A Laboratory Manual, Second Edition, Maniatis et al, Cold Spring Harbor). The genes are also under the control of regulatory elements of DNA so as to provide for their expression. The use of the same regulatory elements for both chains will provide substantially balanced expression.

Construction of the transfer vectors may be carried out in accordance with procedures known in the art (Molecular Cloning: A Laboratory Manual, Second Edition, Sambrook et al, Cold Spring Harbor). Transfection of an appropriate cell line with the transfer vector(s) may be carried out simply by standard transfection procedures, such as calcium phosphate precipitation or preferably lipofectin.

The cell lines used according to the invention for the production of the antibody may be cultured using standard procedures for the cell lines in question. The recombinant antibody produced may be purified and formulated in accordance with standard procedures.

The production of recombinant antibodies by the method according to the invention using a recombinant vaccinia virus has a number of advantages particularly in terms of versatility and speed. As noted above, vaccinia virus will infect a wide range of cells and the method according to the invention can thus be used to produce recombinant antibodies in a wide range of different types of [illegible]

[illegible] present invention means that it will usually be possible to select

a type of cell that carries out the processing necessary to produce a fully functional antibody. The method according to the present invention also has the advantage of speed in that good levels of expression of the recombinant antibody can be achieved directly without the need for the successive rounds of amplification which are necessary in certain other expression systems. Cell lines suitable for production of a recombinant antibody can thus be derived conveniently and quickly.

In the accompanying drawings:

FIGURE 1 shows a transfer vector designated p1112 used to construct recombinant vaccinia viruses expressing either the heavy chain or the light chain of antibody Campath 1H. The vector was obtained from M. Mackett (Manchester, U.K.) and contains a cloning region identified by the Hind III site under control of the vaccinia virus 7.5k promoter. This region contains sites for restriction enzymes Stu 1, BamH1, Sma 1, Hind III and EcoR1 followed by translational stop codons. The gpt gene under control of the 19k vaccinia virus promoter allows for positive selection of recombinants. Flanking sequences of the vaccinia virus thymidine kinase gene (Tk_L and Tk_R) allows for recombination with wild type ("wt") virus. The Hind III (deleted)/BamHI (deleted) fragment is derived from pBR328. The coding sequences for heavy and light chain proteins are carried on Hind III fragments (sites indicated by brackets).

FIGURE 2 shows a western blot of proteins from recombinant vaccinia virus infected HTK- cells performed as described below. Proteins were separated by SDS/PAGE on 12.5% gels and transferred to nitrocellulose. The blots were analysed using mixed anti-heavy chain serum (1:250) and anti-light chain serum (1:1000). Lanes: 1, H-chain recombinant

infected H+L recombinant [supernatant]; 8, wt vaccinia virus [cell]; 9, wt vaccinia virus [supernatant].

FIGURE 3 shows a further western blot of proteins from recombinant vaccinia virus infected HTK$^-$ cells performed as described below. Proteins were separated by SDS/PAGE on 12.5% gels and transferred to nitrocellulose. The blots were analysed using a mixed anti-heavy chain serum (1:500) and anti-light chain serum (1:1000). A. Non-reducing gel. Lanes: 1, Protein-A bound material from supernatant of H+L recombinant dual infected cells; 2, supernatant of H+L recombinant dual infected cells; 3, supernatant of H+L recombinant dual infected cells; 4, Campath 1H control 1μg; 4, wt vaccinia virus infected supernatants; 5, wt vaccinia virus infected supernatants; B. Reducing gel. Lanes: 1, Protein-A bound material from supernatant of H+L recombinant dual infected cells; 2, Before Protein-A selection; 3, Campath 1H control 1μg; 4, wt vaccinia virus infected supernatants.

FIGURE 4 shows a quantitation of Protein-A purified material from recombinant vaccinia virus infected HTK$^-$ cells by western blot performed as described below. Proteins were separated by SDS/PAGE on 12.5% gels and transferred to nitrocellulose. The blot was analysed using mixed anti-heavy chain serum (1:1000) and anti-light chain serum (1:1000). The migration of the recombinant vaccinia virus Campath 1H heavy chain has been affected by the large amount of carrier BSA present in the samples. Lanes: 1, Campath 1H control 0.5μg; 2, Campath 1H control 0.1μg; 3, Recombinant vaccinia virus Campath 1H 20μl; 4, Recombinant vaccinia virus Campath 1H 200μl.

The following Examples are provided in illustration of the present invention.

EXAMPLE

[illegible] monoclonal were originally grafted directly into genomic human heavy

and light chain frameworks (Riechmann et al, Nature, 1988, 322, 323-327). These constructs were engineered for expression in the myeloma cell line YO. The myeloma cell line TF57 (Hale et al, ibid,) was used to generate size selected cDNA fractions of 0.9-1.2kb and 1.4-1.7kb for the light and heavy chain cDNAs respectively. These were used to make EcoR1 linkered cDNA libraries in λgt10. All procedures were as described by Huynh et al (DNA Cloning, Vol I: A Practical Approach, 1984, Glover, D (Editor), IRL Press, Oxford). The libraries were screened using ^{32}P nick translated probes specific for the variable regions to isolate full length cDNA clones. For the light chain cDNA, the 5' untranslated leader was removed up to position -32 using Bal-31 exonuclease and a HindIII linker added. For the 3' end, use was made of a unique SacI site 47bp upstream of the stop codon. A SacI-HindIII oligonucleotide pair was used to regenerate this sequence and position the HindIII site immediately after the stop codon. For the 5' end of the heavy chain cDNA, the unique NcoI site overlapping the ATG start codon was used to re-build a 29bp untranslated leader, identical to that of the light chain, using a HindIII-NcoI oligonucleotide pair. At the 3' end, the unique NaeI site 12bp downstream of the stop codon was converted into a HindIII site using linkers.

EXAMPLE 2 Construction of recombinant vaccinia viruses containing the heavy and light chain genes of the antibody Campath 1H.

The transfer vector used in this study is shown in Figure 1. cDNA clones corresponding to the heavy or the light chain of Campath 1H antibody were as produced according to Example 1. These clones had NcoI sites introduced at their initiation codons and could be excised from the host plasmid as HindIII fragments. These HindIII fragments were transferred separately to the vaccinia virus transfer vector to

Vaccinia virus (strain WR)., originally from the A.T.C.C. was replicated in HeLa thymidine kinase-minus 143 cells (HTK⁻ cells). Cells were grown in MEM with 10% foetal bovine serum.

Recombinant vaccinia viruses were prepared basically as described (Mackett, Seminars in Virology, (1990), 1, 39-47) with the following modifications. Lipofectin was used instead of calcium phosphate to introduce DNA into cells. Lipofectin was used according to the manufacturers instructions (GIBCO - BRL). Briefly 90% confluent monolayers of HTK- cells were infected at an m.o.i. of 0.1 pfu/cell with vaccinia virus (WR). After incubation for 1h at 37°C the virus inoculum was removed and replaced with serum free opti-MEM medium (GIBCO-BRL) after washing the cells. Lipofectin-DNA (10μg in 100μl) was added gently with mixing and incubated for 24h at 37°C. The medium was then replaced with 5ml MEM containing 5% foetal calf serum and incubated a further 24h, after which virus was released from cells by freezing and thawing.

EXAMPLE 3 Identification of recombinant vaccinia viruses expressing heavy or light chains

The transfer vectors contain the E. coli guanine phosphoribosyltransferase gene and this allows for positive selection of recombinant viruses by growth in the presnce of mycophenolic acid (MPA). Confluent monolayers of HTK⁻ cells were incubated at 37°C for a minimum of 2h in selection medium containing 2.5μg/ml mycophenolic acid (MPA), 250μg/ml xanthine and 15μg/ml hypoxanthine. The selection medium was removed and replaced with dilutions of virus harvested from the transfection.

After 1h at 37°C the virus inoculum was replaced with selection medium and the infected monolayers incubated at 37°C for 36 - 48 h. The

plating under agarose in selective medium for a further two rounds

Virus harvested from the transfected cells was subjected to MPA selection at dilutions of 10^{-1}, 10^{-2} and 10^{-3}. Because it had previously been observed that it was difficult to identify plaques under agarose during the first round of MPA selection, plaquing was done under liquid. Plaques were allowed to develop at 37°C for 36 to 48h. The liquid media was then replaced with 1% agarose containing the MPA selective media and neutral red to visualize plaques. Plaques were picked into 1ml of diluent and further plaque purified one or two times under agarose at dilutions up to 10^{-3}. Plaques were always picked at the highest dilution possible.

An immunoplaque assay was used to identify individual recombinant vaccinia viruses expressing either the heavy chain or the light chain. Confluent monolayers of HTK- cells in 3cm wells were infected with approximately 30 pfu of recombinant virus. After 48h the medium was removed and the cell sheets fixed with methanol for 5 mins at room temperature. The methanol was then removed and the cells sheets allowed to air dry. The dry cell sheets were rehydrated by incubation in PBS for 15 mins and then incubated in diluent (1% BSA, 5% FCS in PBS) for 1h at room temperature followed by a 1/500 dilution of either goat anti-human IgG (γ-chain specific) peroxidase conjugate (Sigma A6029) or goat anti-human kappa light chain peroxidase conjugate (Sigma A7164). After incubation for 2h at room temperature the cell sheets were extensively washed with PBS before incubation with substrate (3,3-diaminobenzidene tetrahydrochloride 0.1mg/ml; H_2O_2 0.1% in PBS).

Most of the recombinants selected by MPA reacted with the expected antibody. Uninfected or wild type vaccinia virus (WR) infected cells did not give a signal; neither did heavy chain recombinants react with anti-light chain antibody or vice versa. It is of interest to note

EXAMPLE 4 Synthesis and location of proteins in single and dual infected cells

Confluent monolayers of HTK$^-$ cells were infected with either the heavy chain or the light chain vaccinia virus recombinant or both at a m.o.i. of 5 pfu/cell for each virus. Infected cells were incubated in opti-MEM medium in the absence of serum. After 24h the cells and the media were harvested and separated by centrifugation. Samples of whole cells and the clarified medium were analysed by PAGE and western blotting (see Fig 2).

Total cell lysates and cell supernatants were subjected to SDS/PAGE according to Laemmli (Nature (London), (1970), 227, 680-685) using 12.5% gels. Where appropriate, supernatants were concentrated by precipitation with 2 volumes of acetone at -20°C for 16h. Proteins were transferred to nitrocellulose and western blot analysis carried out as follows using the anti-sera described in the immune plaque screening procedure (Example 3).

Nitrocellulose blots were first incubated in blocking buffer (5% milk solids in PBS) for 1h. Blots were then incubated in the appropriate sera diluted in blocking buffer. For most experiments the anti-heavy chain and anti-light chain peroxidase conjugates were used together at dilutions indicated in the description of the figures given above. After 2h the sera were removed and the blots washed extensively in PBS. Blots were developed using diaminobenzidene tetrahydrochloride, 0.1mg/ml; 0.1% H_2O_2 as substrate.

Cells infected with the heavy chain recombinant synthesized a protein of the correct size which reacted with an anti-heavy chain specific antibody. This protein was associated with the cell fraction and none was detected in the medium. [illegible]

contrast to the heavy chain, the light chain was found in both cells

and medium (Fig 2, lanes 3 & 4). Furthermore, the secreted light chain was slightly smaller than the intracellular protein, which is consistent with the cleavage of a leader peptide upon secretion. The observation that the majority of the light chain was secreted explained the finding from the immunoplaque assay (which only measures intracellular proteins) that there was more heavy chain than light chain in the cells.

When cells were dual infected both heavy and light chains were synthesized. The light chain behaved as before with about two thirds being processed and secreted. However the heavy chain, in contrast to the single infection, was now detectable in the cell free medium (Fig 2, lane 7). Processing of the heavy chain leader was not apparent, although the predicted difference in molecular weight would be difficult to see with the gel system used. Approximately one fifth of the heavy chain was secreted and it would appear that this was a consequence of the co-expression of both heavy and light chains. The implication of this was that the heavy and light chains secreted into the medium were associated and further experiments were performed to confirm this.

EXAMPLE 5 Association of secreted heavy and light chains

Two approaches were used to establish whether the heavy and light chains secreted into the medium of dual infected cells were associated. The first, was to analyse the proteins on non-reducing SDS gels. The heavy and light chains of an antibody are held together by disulphide bonds and migrate as a complex of approximate molecular weight 150 kDa on non-reducing SDS gels. The second approach was to utilize the protein A binding property of the constant region of heavy chain. If the light chain was associated with the heavy chain then it

Firstly, the proteins from dual infected cell culture medium were electrophoresed under non-reducing conditions and analysed by western blotting. The control sample of Campath 1H antibody migrated as a high molecular weight complex at the top of the gel (Figure 3a, lane 4). Samples from cultures co-infected with the heavy and light chain recombinant vaccinia viruses also showed material migrating as a high molecular weight complex. However, although all the heavy chain monomers were associated with light chains, the converse was not true since light chain monomers were seen (Figure 3a, lanes 2, 3). This observation was consistent with the obvious molar excess of light chain in the supernatant.

Secondly, proteins capable of binding to protein A-sepharose were analysed by western blotting. Figure 3b shows the total proteins present in the culture medium of dual infected cells and those which bound to protein-A (Figure 3b, lanes 1,2). It can be seen that most (probably all) of the heavy chain and a proportion of the light chain bound to protein A. Since light chain alone does not bind to protein A the binding seen is indicative of the association of light chain to heavy chain. The molar ratio of heavy and light chains bound to protein A was 1:1 as expected. When electrophoresed on non-denaturing gels the protein A bound material migrated as a high molecular weight complex and there were no monomeric heavy or light chains (Figure 3a, lane 1).

EXAMPLE 6 Functional assay of recombinant antibody

Protein A binding heavy and light chain complex from the supernatant of recombinant vaccinia virus infected cells was assayed for activity in two different assays and compared to myeloma derived antibody. The supernatant from dual infected cells was made virus free by

material was analysed by SDS-PAGE and western blotting to assess the

total amount of heavy and light chain present (Figure 4). Based on this information it was estimated that the concentration of combined heavy and light chain was in the range 0.5 to 1μg/ml. This material was then analysed for functional activity in a T-cell binding assay and a purified antigen binding assay.

The concentration of antibody was measured by an ELISA system using purified non-recombinant Campath 1H as a standard. Antibody was captured using goat anti-human IgG (heavy and light chain) sera and detected using goat anti-human IgG (heavy chain specific) peroxidase conjugated sera using tetra methylbenzidine as substrate.

Antibodies were tested for functional activity by two methods. Firstly, to assess T-cell binding, human T lymphocytes, which express the Campath 1H antigen, were coated onto the surface of 96 well flat bottomed plates. Test samples of antibody were then added to the plates and, after incubation, excess antibody was removed by extensive washing. Bound antibody was measured by binding of I125 protein-A and, after removal of unbound excess I125 protein-A, the radioactive counts in each well were measured. Results are expressed as μg/ml of active protein relative to a standard.

The second functional assay measured the binding of expressed antibody to extracted antigen. The Campath 1H antigen was prepared from human cells by chloroform/methanol/water extraction and used to coat 96 well flat bottomed plates. Antibody samples were then added to the plates and, after incubation, excess antibody was removed by extensive washing. Bound antibody was measured by using an alkaline phosphatase labelled goat anti-human (chain specific) serum. Results are expressed as μg/ml of active protein relative to a standard.

concentration of antibody as [illegible] μg/ml. It agreed well with the estimate from western blots. The two functional assays showed that the vaccinia

virus derived materials had between 60-70% of the activity of the control Campath sample. This was considered to be satisfactory for two reasons. Firstly there is no absolute standard available for Campath 1H antibody at present and secondly, it is known to lose activity upon storage, since ideal conditions have not yet been established.

As described in the above experiments, the expression levels of heavy and light chains are about 1-2μg per 106 cells, and between 10-20% of this is secreted as assembled antibody. These proteins are under the control of the vaccinia virus 7.5K promoter and it should be possible to improve yields, for example by using the EMCV leader and T7 polymerase enhancing systems.

TABLE 1

Functional activity of recombinant activity

Assay	Concentration μg/ml protein relative to standard	Relative activity as percentage of standard
ELISA	0.65	100
T-cell binding	0.34	61
Antigen binding	0.40	71

CLAIMS

1. A recombinant vaccinia virus capable of expressing on infection of a suitable cell the light chain and/or the heavy chain of an antibody.

2. A virus as claimed in Claim 1 wherein the antibody is a chimaeric or humanised (CDR-grafted) antibody.

3. A virus as claimed in Claim 2, wherein the antibody is directed against an antigen selected from CD2, CD3, CD4, CD5, CD7, CD8, CD11a,b,c, CD18, CD19, CD25 and CDw52.

4. A virus as claimed in Claim 1 wherein the antibody is a humanised (CDR-grafted) antibody against the CDw52 antigen.

5. A virus as claimed in any of Claims 1 to 4 which contains DNA encoding the light chain and/or the heavy chain of the antibody under control of a suitable promoter.

6. A virus as claimed in Claim 5 wherein the promoter is selected from the vaccinia virus 7.5k, 11k and 19k promoters.

7. A virus as claimed in Claim 5 or 6 which either contains DNA encoding the light chain of the antibody or DNA encoding the heavy chain of the antibody.

8. A virus as claimed in Claim 5 or 6 which contains DNA encoding both the light chain and the heavy chain of the antibody, either under control of the same promoter or under control of separate promoters.

9. A method of preparing a virus as claimed in any of Claims 1 to 8 comprising infecting suitable cells with wild type vaccinia virus and transfecting the infected cells with a transfer vector containing DNA

encoding the light and/or heavy chain of the antibody under control of a suitable promoter, DNA encoding a selectable marker also under control of a suitable promoter and sufficient vaccinia virus DNA to allow for recombination with the wild type vaccinia virus; (iii) harvesting vaccinia virus from the transfected cells; (iv) re-infecting further suitable cells with the harvested vaccinia virus; (v) selecting re-infected cells by means of the selectable marker; and (vi) harvesting recombinant vaccinia virus from the selected cells.

10. A process as claimed in Claim 9 wherein the selectable marker is the E. coli guanine phosphoribosyltransferase gene.

11. A process for the production of a recombinant antibody which comprises culturing cells which are infected with a recombinant vaccinia virus capable of expression of the light chain and the heavy chain of the antibody or co-infected with recombinant vaccinia viruses capable of expression of the light chain and the heavy chain respectively of the antibody, and recovering the antibody from the culture medium.

12. A process as claimed in Claim 11 wherein the cells are HeLa(TK-) cells or a B cell line.

13. A process as claimed in Claim 11 or 12 wherein the antibody is a chimaeric or humanised (CDR-grafted) antibody.

14. A process as claimed in Claim 13 wherein the antibody is directed against an antigen selected from CD2, CD3, CD4, CD5, CD7, CD8, CD11a,b,c, CD13, CD19, CD25 and CDw52.

16. A cell line capable of expressing a recombinant antibody, characterised in that the cell line is infected with a recombinant vaccinia virus capable of expression of both the light chain and the heavy chain of the antibody or co-infected with recombinant vaccinia viruses capable of expression of the light chain and the heavy chain respectively of the antibody.

17. A cell line as claimed in Claim 16 wherein the antibody is a chimaeric or humanised (CDR-grafted) antibody.

18. A cell line as claimed in Claim 17 wherein the antibody is directed against an antigen selected from CD2, CD3, CD4, CD5, CD7, CD8, CD11a,b,c, CD18, CD19, CD25 and CDw52.

19. A cell line as claimed in Claim 16 wherein the antibody is a humanised (CDR-grafted) antibody against the CDw52 antigen.

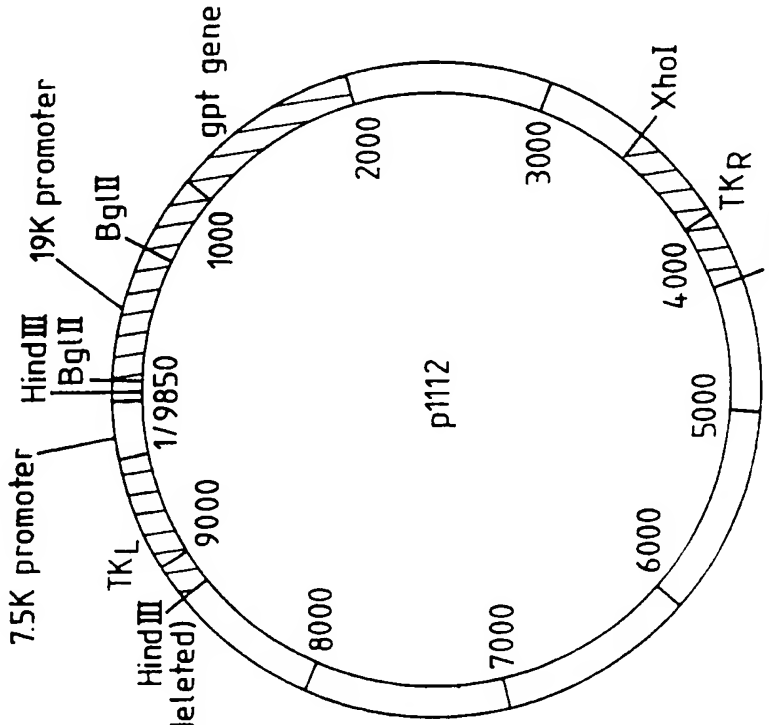


Fig.1.

AVY CHAIN

GCTTTACAGTTACTGAGCACACAGGACCTCACC ATG GGA ..... AAA TGA TGCGACGGCCAAGCTT
met gly lys *

HT CHAIN

GCTTTACAGTTACTGAGCACACAGGACCTCACC ATG GGA ..... TGT TAG AAGCTT
met gly cys *

Fig. 2.

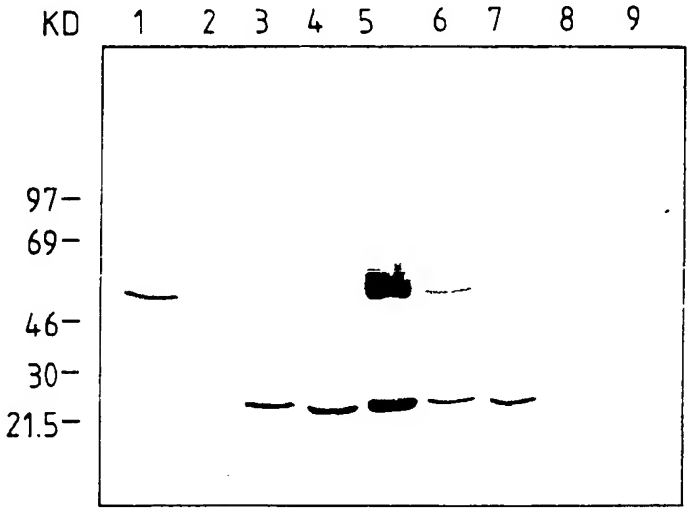


Fig. 4.

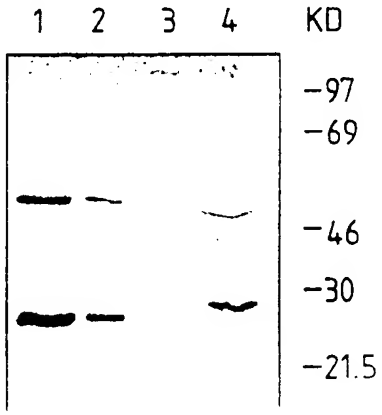
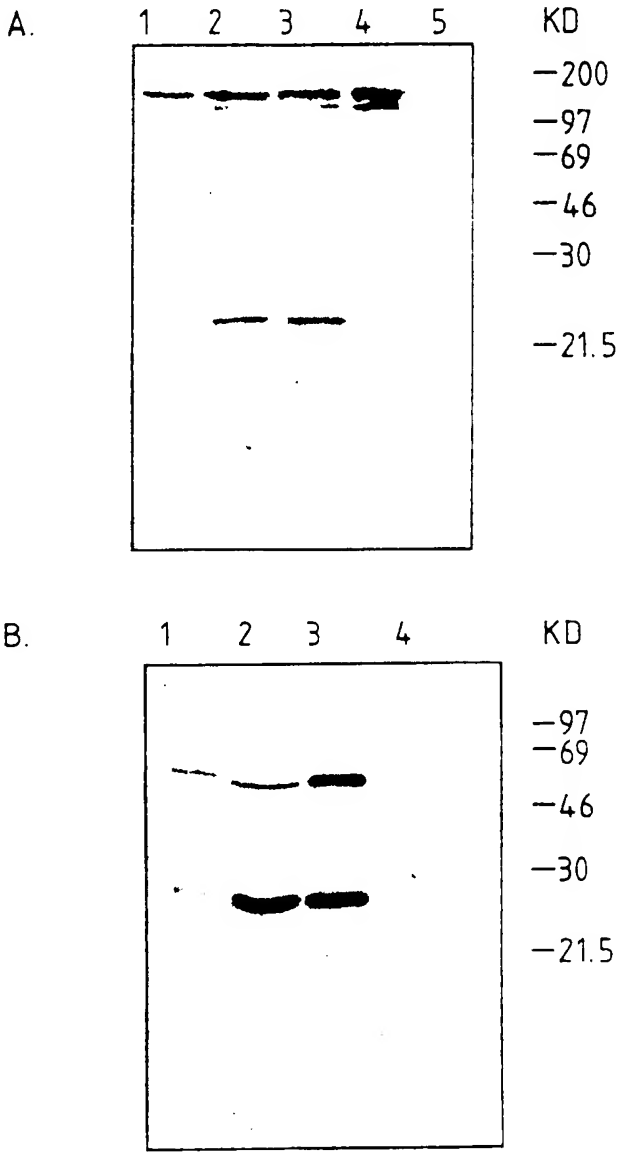


Fig.3.



INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/01170

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/86; C12N15/13; C12N5/10

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C12P ; C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	TRENDS IN BIOTECHNOLOGY vol. 8, no. 1, January 1990, CAMBRIDGE pages 20 - 25 J.N.MINER AND D.E.HRUBY 'Vaccinia virus: a versatile tool for molecular biologists' see page 24, column 3, paragraph 3 ---	1-19
Y	EP,A,0 328 404 (MEDICAL RESEARCH COUNCIL) 16 August 1989 see page 4, line 37 - line 44 ---	1-19
A	BIOTECHNOLOGY vol. 8, no. 12, July 1990, NEW YORK US pages 651 - 654 J.ZU PULITZ ET AL. 'Antibody production in baculovirus-infected insect cells' see the whole document -----	1-19

° Special categories of cited documents :[10]

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	CUPIDO M.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9201170 SA 61393

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 01/10/92

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		WO-A-	8907452	24-08-89
		GB-A,B	2216126	04-10-89